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Review

# The $Na^+$-dependence of alkaliphily in *Bacillus*

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**Abstract**

A $Na^+$ cycle plays a central role in the remarkable capacity of aerobic, extremely alkaliphilic *Bacillus* species for pH homeostasis. The capacity for pH homeostasis, in turn, appears to set the upper pH limit for growth. One limb of the alkaliphile $Na^+$ cycle consists of $Na^+/H^+$ antiporters that achieve net $H^+$ accumulation that is coupled to $Na^+$ efflux. The major antiporter on which pH homeostasis depends is thought to be the Mrp(Sha)-encoded antiporter, first identified from a partial clone in *Bacillus halodurans* C-125. Mrp(Sha) may function as a complex. While this antiporter is capable of secondary antiport energized by an imposed or respiration-generated protonmotive force, the possibility of a primary mode has not been excluded. In *Bacillus pseudofirmus* OF4, at least two additional antiporters, including NhaC, have supporting roles in pH homeostasis. Some of these additional antiporters may be especially important for antiport at low [$Na^+$] or at near-neutral pH. The second limb of the $Na^+$ cycle facilitates $Na^+$ re-entry via $Na^+$/solute symporters and, perhaps, the ion channel associated with the $Na^+$-dependent flagellar motor. The process of pH homeostasis is also enhanced, perhaps especially during transitions to high pH, by different arrays of secondary cell wall polymers in the two alkaliphilic *Bacillus* species studied most intensively. The mechanisms whereby alkaliphiles handle the challenge of $Na^+$ stress at very elevated [$Na^+$] are just beginning to be identified, and a hypothesis has been advanced to explain the finding that *B. pseudofirmus* OF4 requires a higher [$Na^+$] for growth at near-neutral pH than at very alkaline pH values. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* $Na^+/H^+$ antiporter; $Na^+$/solute symporter; $Na^+$-motive flagellum; Secondary cell wall polymer

## 1. Introduction

A remarkable diversity of extremely alkaliphilic bacteria grow optimally and robustly at pH values of 10–11 and even above [1–4]. As a result of systematic investigations of the prokaryotic and archaeal flora of both man-made enrichments (e.g. processing plants for indigo dye production) and stable natural enrichments (e.g. soda lakes of Africa or Central Asia), by several groups of investigators [1–3], large numbers of such organisms are now available for further study. They can also be compared with other extreme alkaliphiles that are readily isolated from ostensibly non-selective environments [1,4]. A long-recognized correlation between $Na^+$-dependence and alkaliphily [4] is widespread among the recent isolates. There is a general requirement for at least low levels of $Na^+$ by most, and perhaps all, alkaliphilic *Bacillus* studied to date although large variations exist in the concentration of $Na^+$ required [1,3].

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There is also variation in the pH range for extreme alkaliphiles, with some alkaliphiles being restricted to growth at pH values above 9 (obligate alkaliphiles) whereas others can grow at near-neutral, although usually not at neutral, pH (facultative alkaliphiles). The pH range of growth is dependent upon the growth substrate of aerobic alkaliphiles, with fermentable growth substrates supporting a pH range that is down-shifted relative to that of the same strain grown on non-fermentative carbon sources such as malate [1,5,6]. Among the extremely alkaliphilic microorganisms, there are numerous strains that are also highly halophilic and a smaller, but growing, number of examples of thermophiles [1,3]. In order to focus this review on recent findings that relate specifically to alkaliphily, rather than to multiple extreme conditions, the discussion will center on two facultatively alkaliphilic *Bacillus* species, *Bacillus pseudofirmus* OF4 and *Bacillus halodurans* C-125. These are the alkaliphiles in which the dependence upon $Na^+$ has been studied in greatest detail, especially as it relates to extreme alkaliphily itself. Both *B. pseudofirmus* OF4 (formerly *Bacillus firmus* OF4) and *B. halodurans* C-125 (formerly *Bacillus lentus* C-125) have recently been re-classified as a result of new studies [7,8]. These efforts were undertaken inasmuch as the imminent completion of the *B. halodurans* C-125 genome sequence project [9,10] makes the precise identification of this strain and its relationship to other extremely alkaliphilic *Bacillus* strains (as well as to non-alkaliphilic *Bacillus* strains with sequenced genomes) particularly timely.

This review will provide a status report on the active transporters that are involved in $Na^+$-dependent pH homeostasis. They constitute a $Na^+$ cycle that also contributes to solute uptake and to motility. Examples of secondary cell wall polymers that apparently support pH homeostasis, perhaps especially during upward transitions in pH, will also be reviewed. A diagrammatic summary of these elements is shown in Fig. 1. The summary notes (with question marks) some of the unresolved questions that are currently being investigated in several laboratories, including our own. Briefly, at the top of the diagram, electron transport complexes and the menaquinone of the proton-translocating respiratory chain are shown. Respiration has been shown to be coupled to $H^+$ translocation but, to date, not to $Na^+$ translocation, in the non-halophilic alkaliphilic *Bacillus* species [11]. Alternate terminal oxidases have been demonstrated including *cta*-, *aco*- and *bd*-types [12,13] as well as a proposed alternate oxidase that has not yet been completely characterized [14]. Thus far, an energy-coupled complex I, NADH:(mena)-quinone oxidoreductase, has not been definitely shown in *B. halodurans* C-125, *B. pseudofirmus* OF4 or any of the other alkaliphilic *Bacillus* species whose respiratory chains have been studied in some detail [11,13]. Greater detail and opportunity for study is now created by the completion of the genome sequence of *B. halodurans* C-125 [15]. If a complex I is found in an alkaliphile, it will be of particular interest to probe whether it might translocate $Na^+$ in addition to $H^+$ in view of the recent findings of Dimroth and colleagues in enteric bacteria [16,17]. However, it may be particularly important for alkaliphiles such as *B. pseudofirmus* OF4 and *B. halodurans* C-125 to tightly control any primary $Na^+$ pumping so that it occurs only under conditions of $Na^+$ excess.

Cytoplasmic pH homeostasis, i.e. establishment of a pH gradient, acid in, occurs during aerobic growth of respiring, $H^+$-extruding cells or in cells growing on fermentative substrates and using the $H^+$-coupled F-ATPase to extrude protons. The ATPase is depicted next to the respiratory chain in Fig. 1. The pH homeostasis mechanism that facilitates net proton accumulation depends upon a group of $Na^+/H^+$ antiporters, depicted as a Mrp complex and NhaC in Fig. 1. These antiporters couple net $H^+$ uptake with $Na^+$ extrusion [5,6,18]. Unlike pH homeostasis in non-alkaliphilic *Bacillus subtilis*, which can be coupled to $K^+$ efflux, pH homeostasis in the aerobic extreme alkaliphiles is exclusively coupled to $Na^+$ (perhaps to avoid detrimental reductions in the cytoplasmic $[K^+]$) [6,18]. Thus the alkaliphiles' capacity for $Na^+$ extrusion may have to be protected for this crucial function under conditions other than $Na^+$ stress. In fact, routes that make cytoplasmic $Na^+$ available, i.e. complete the $Na^+$ cycle, are necessary for pH homeostasis independently of other functions to which they relate. Shown in the diagram, are $Na^+$ re-entry through $Na^+$/solute symporters and through the $Na^+$-motive flagellar apparatus [4,5,19,20]; another hypothetical $Na^+$ re-entry route that is noted in Fig. 1, is existence of $Na^+$ channels that resemble

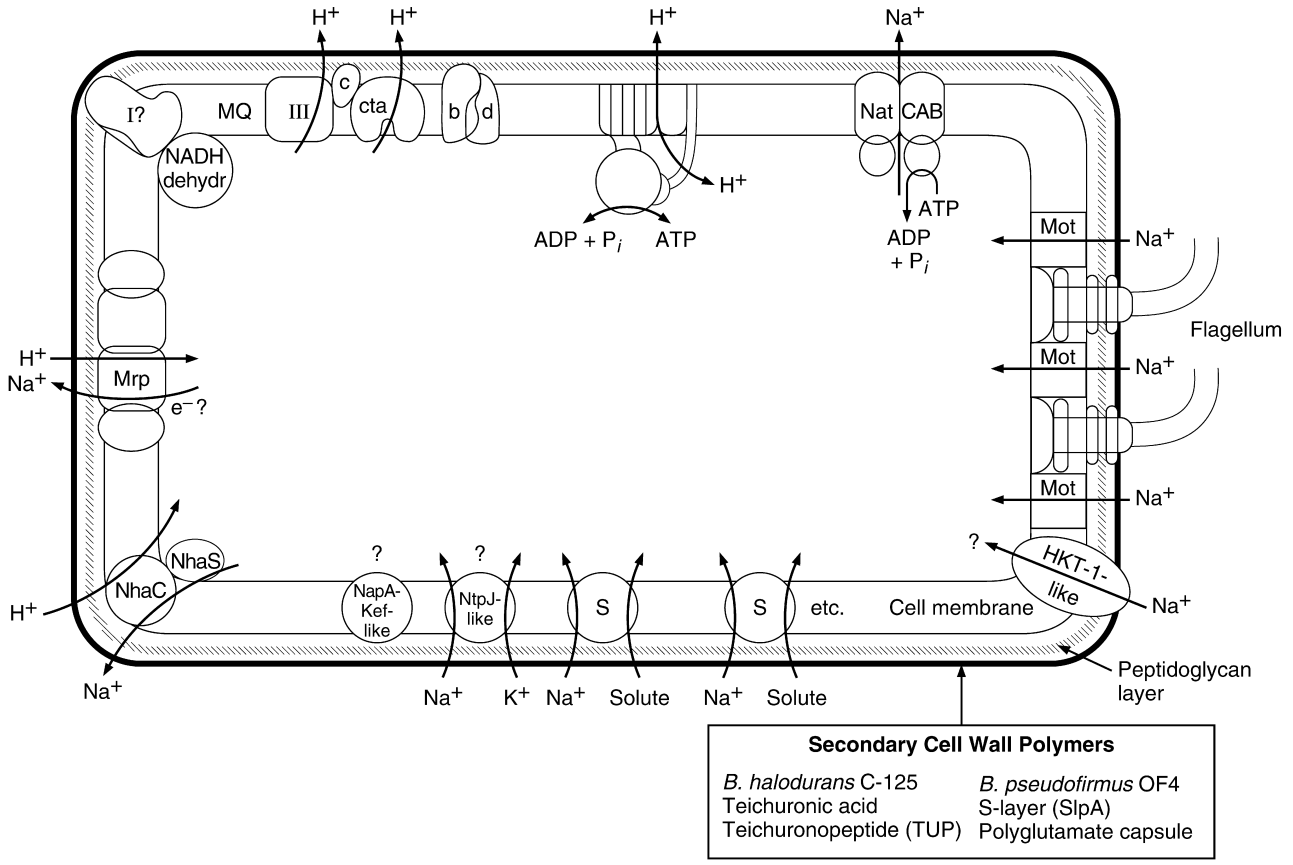


Fig. 1. A diagrammatic summary of the primary ion extrusion mechanisms, the elements of a $Na^+$ cycle, and secondary cell wall polymers that have demonstrated or hypothesized roles in $Na^+$-dependent pH homeostasis, solute uptake, or motility in extremely alkaliphilic *Bacillus* species. See the text for discussion.

the plant HKT1 protein [21] that has putative homologs in bacteria. Undoubtedly there are also additional $Na^+$ extrusion mechanisms in these facultative alkaliphiles. *B. pseudofirmus* OF4, for example, grows at [$Na^+$] values above 1 M and must accommodate to $Na^+$ stress [22]. One system for $Na^+$ extrusion that is apparently not coupled to $H^+$ entry is the ABC-type $Na^+$ extrusion system, NatCAB [23], shown in the diagram. There may well be more. Finally, secondary cell wall polymers have been found to contribute to alkaliphily [24–26]; those that have been identified or for which genetic loci have been found in either *B. halodurans* C-125 or *B. pseudofirmus* OF4 are listed in the diagram in Fig. 1.

Before focusing further on the elements depicted in Fig. 1, it should be noted that the developing understanding of these elements is greatly assisted by work on other extreme alkaliphiles – including the halo- and sensory-rhodopsin-containing archaebacterium, *Natronobacterium pharaonis* [27,28] and anaerobic alkaliphiles [29,30] – and alkaline-tolerant bacteria [21,27,31–34] that solve the challenge of high pH by different but overlapping assortments of these and other elements.

## 2. $Na^+$ cycle and its roles in pH homeostasis, solute uptake and motility

### 2.1. *pH homeostasis: the central challenge of alkaliphily involves $Na^+/H^+$ antiporters and $Na^+$ re-entry systems*

By the 1980s, non-alkaliphilic mutants from several different alkaliphilic strains had made it evident that there was a relationship between alkaliphily and activity of $Na^+/H^+$ antiport and the whole active $Na^+$ cycle [1,4–6]. While it was recognized early

that an extraordinary capacity for pH homeostasis was a necessary feature of extreme alkaliphiles, it was not clear whether it was this capacity that determined the upper pH limit for growth (e.g. as opposed to alkali-stability of key cellular components exposed to the outside milieu). Nor was there information about how the alkaliphilic *Bacillus* species' capacity for pH homeostasis differed qualitatively or quantitatively from that of non-alkaliphilic *Bacillus* species. Comparative assessments of the total $Na^+/H^+$ antiporter activity of membranes from *B. subtilis* and *B. pseudofirmus* OF4, using respiration-dependent $^{22}Na^+$ efflux by membrane vesicles as the assay, subsequently indicated that the aggregate rate in the alkaliphile was at least 10-fold greater than that of the non-alkaliphile. The determinations were made under comparable conditions that were slightly suboptimal for both strains [35]. A qualitative difference in the specificity of the monovalent cation requirement for pH homeostasis was also found. During rapid upward shifts in the external pH from 8.5 to 10.5 in malate-containing media, pH 8.5-equilibrated cells of *B. pseudofirmus* OF4, but not of *B. subtilis*, maintained a steady cytoplasmic pH at about pH 8.3. This cytoplasmic pH regulation depended upon the presence of $Na^+$. $K^+$ was not efficacious. Moreover, when a more modest challenge was imposed, i.e. a shift of pH 7.5-equilibrated cells to an external pH of 8.5, cells of both *B. subtilis* and *B. pseudofirmus* OF4 maintained a cytoplasmic pH of about 7.5 but *B. subtilis* required either $Na^+$ or $K^+$ (in choline, the cytoplasmic pH rose to 8.5), whereas pH homeostasis in *B. pseudofirmus* OF4 still required $Na^+$ specifically [18,35]. These comparisons suggest that alkaliphilic *Bacillus* species possess a much greater aggregate activity of $Na^+/H^+$ antiport than *B. subtilis* and that the alkaliphile specifically uses $Na^+/H^+$ antiporters for pH homeostasis whereas *B. subtilis* uses a less specific complement of $Na^+(K^+)/H^+$ antiporters. Growth on glucose may spare the full requirement of $Na^+$-dependent pH homeostasis since *B. pseudofirmus* OF4 cells shifted from pH 8.5 to 10.5 in such media can maintain a cytoplasmic pH of 9.5 even without added $Na^+$ [12]. It is not clear whether or not this is antiporter-independent acidification of the cytoplasm relative to the medium, e.g. by cytoplasmic acid production.

The strongest indication that the capacity for pH homeostasis does set the upper pH limit for alkaliphile growth emerged from studies of *B. pseudofirmus* OF4 in continuous cultures on malate-containing medium maintained at carefully controlled pH values that spanned 7.5–11.4 [36]. As the external pH increased from pH 7.5 to 10.5, the cytoplasmic pH never reached 8.5, i.e. a ΔpH, acid in, a bit higher than two full pH units was reached (Fig. 2). The magnitude of that ΔpH did not continue to increase as the external pH was raised further, above pH 10.5. As the cytoplasmic pH rose accordingly, the rate of growth decreased in parallel (shown as doubling time in Fig. 2).

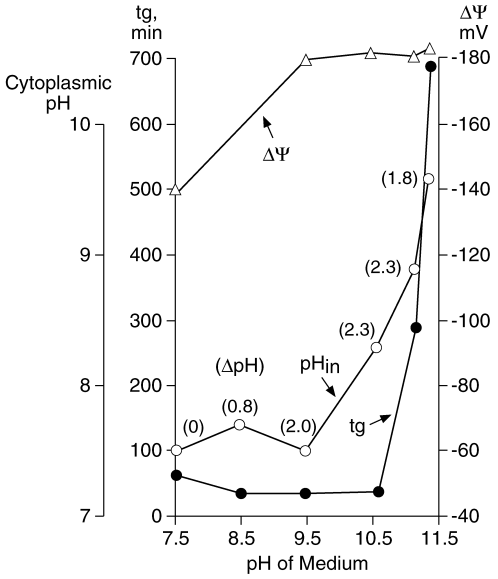


Fig. 2. The profile of cytoplasmic pH, ΔpH, doubling time, and ΔΨ of *B. pseudofirmus* OF4 growing on malate-containing medium in continuous culture at various controlled values of the external pH. The data are taken from the study of Sturr et al. [36] as replotted in [73]. The numbers in the parentheses are the values of the ΔpH, acid in. The growth rate is expressed as doubling or generation time, tg, in minutes.

If secondary $Na^+/H^+$ antiporters completely account for the acidification of the cytoplasm relative to the external pH throughout the pH range for growth, the antiporters must be electrogenic, with a $H^+/Na^+$ ratio greater than unity so that the transmembrane electrical potential (ΔΨ, positive out) can energize [37]. This is a necessity if secondary antiport is to continue to acidify as the pH gradient becomes increasingly adverse to further cytoplasmic $H^+$ accumulation. Essentially all $^{22}Na^+$ extrusion by preloaded cells of *B. pseudofirmus* OF4 was inhibited

either by CCCP or valinomycin+$K^+$ [38]. This suggested that under conventional growth conditions the efflux of $Na^+$ was indeed mediated by electrogenic secondary antiporters in exchange for $H^+$. However, as indicated in Fig. 2, the $\Delta\Psi$, positive out, of cells growing at pH values from 7.5 to 10.5, increases significantly even though increased electrogenic $H^+$ accumulation in exchange for $Na^+$ might be expected to be a major consumer of the $\Delta\Psi$. These kind of data suggest, as had been proposed by Skulachev [39], that extreme alkaliphiles would be best served by involving a primary mechanism for pH homeostasis. As described more recently by Skulachev [28], work by others on archaeal alkaliphile *Natronobacterium pharoanis* is consistent with inward proton movement through an unknown pathway, acidifying the cytoplasm relative to the external medium, secondary to generation of a very high $\Delta\Psi$ by primary, inward $Cl^-$ pumping by halorhodopsin, i.e. generation of a $\Delta\Psi$ by the light-driven halorhodopsin activity [40].

### 2.2. Alkaliphile $Na^+/H^+$ antiporters: the Mrp (Sha) complex and several supporting players

The *mrp* operon of *B. pseudofirmus* OF4 [5,18] and of *B. subtilis* (also called *sha*) [41–44] as well as homologous operons from *Staphylococcus aureus* [45] and *Rhizobium meliloti* [46] encode seven hydrophobic gene products. The first recognized *mrp* fragment was described in *B. halodurans* C-125 as a fragment that crossed over with and corrected a mutation that rendered the organism non-alkaliphilic and unable to acidify its cytoplasm relative to the outside at high external pH [47,48]. The complementing fragment in this breakthrough study contained an incomplete operon encompassing the first three genes. The work established this operon as a prime candidate for the major antiporter system involved in alkaliphile pH homeostasis, which has since been sequenced in entirety in *B. pseudofirmus* OF4 [5,18]. In the initial work in *B. halodurans* C-125 [48], the first gene of the operon was shown to be the site of the corrected mutation. A second point mutant, in the third gene of the operon, retained antiport activity but was still non-alkaliphilic, leading to the proposal that the first gene encodes a major antiporter in alkaliphily and other genes in the fragment encoded other, perhaps regulatory, activities required for alkaliphily [48–50]. The work to date on this alkaliphile, however, is limited by its exploration of only part of the operon and use of point mutants whose functional leakiness and polar effects are unknown. Targeted in-frame deletions have not yet been made in *B. halodurans* C-125 to facilitate study of the resultant phenotypes and complementation patterns. Such work is currently being undertaken in *B. pseudofirmus* OF4, where the requisite technical approaches have been successfully applied to other antiporter-encoding genes [51].

Important information on this antiporter-encoding locus of alkaliphilic *Bacillus* species has been obtained from studies of *mrp* homologs from neutralophilic prokaryotes. Studies have been conducted on the full operons of *B. subtilis mrp* [41,42,44] and of *S. aureus mnh* [45]. These studies suggest that while the first gene, *mrpA* or *mnhA*, is required for $Na^+/H^+$ antiport activity and is a good candidate for 'a critical structural gene' for antiport, it is not sufficient. The other gene products of the operon, possibly all six of them, are required for the $Na^+$-resistance. Targeted mutants of *B. subtilis* with a disruption of *mrpA*, exhibit a profoundly $Na^+$-sensitive phenotype, a partial loss of $Na^+$- and $K^+$-dependent pH homeostasis in a pH shift experiment, and a decrease in the protonmotive force-dependent $Na^+/H^+$ antiport [41,42]. Thus, as suggested by the work in the *B. halodurans* C-125, this is an important gene. But deletions in the other *mrp* genes, including in-frame, non-polar deletions [41,44], also yield the same level of $Na^+$-sensitivity and loss of aggregate $Na^+/H^+$ antiport. Similarly, analysis of subclones of the full *mnh* operon of *S. aureus* as expressed in an antiporter-deficient *E. coli* strain [45] are consistent with a requirement for the entire operon for complementation of that strain with respect to $Na^+$-resistance and antiport. Several additional points of importance have emerged from these studies: (i) in each instance, the *mrp*/*sha*/*mnh* locus encodes a $Na^+/H^+$ antiporter that can be energized by a protonmotive force, i.e. it behaves like a typical secondary, electrogenic antiporter with respect to energization by imposed protonmotive force, $\Delta$pH or diffusion potential [41,42,45]; (ii) at least in some of the homologs, the antiport is likely to extend to $Li^+$ [45] and/or $K^+$ [41] or even be primarily a $K^+/H^+$ antiport (as in the *pha*

of *R. meliloti*, [46]); and (iii) there are probably additional transport activities catalyzed by the full operon, as shown for MrpF-dependent ($Na^+$)cholate efflux in *B. subtilis* [41,44].

Does the requirement for multiple gene products for $Na^+$-resistance and antiport reflect a large functional complex and might that, in turn, reflect a capacity for primary energization in addition to the demonstrated secondary energization? These are the critical questions to be answered with respect to this important and novel locus. In the alkaliphile, as indicated above, a capacity for primary energy-coupling could resolve the conundrum of a $\Delta\Psi$ that rises at the external pH values at which electrogenic antiport is ostensibly most active and indispensable (Fig. 2). The possibility of some kind of novel coupling to electron transport is suggested by the long-recognized sequence similarity of many of the genes of this operon to membrane-embedded subunits of the energy-coupled NADH:quinone oxidoreductases of both prokaryotes and eukaryotes (and the absence of an obvious ATP binding site), but there is no evident NADH binding site, nor demonstrable NADH oxidase activity [5,42]. Comparative studies are now underway in our laboratory of the full length *mrp* operons of *B. subtilis* and *B. pseudofirmus* OF4 expressed in different mutant strains of *E. coli*. Such studies should facilitate a more complete dissection of the total energetic profile of the complex. Were Mrp to indeed have a primary energization mode, we hypothesize that this mode would involve partial reactions such that initial electrogenic $Na^+$ efflux is followed by the net $H^+$ accumulation; this would explain the earlier findings in *B. pseudofirmus* OF4 that $Na^+$ efflux was not observed in logarithmically-growing cells, under normal high pH conditions, upon abolition of the $\Delta\Psi$ by valinomycin+$K^+$ [38].

In *B. pseudofirmus* OF4, an additional gene is thus far known to encode a secondary, electrogenic $Na^+/H^+$ antiporter, i.e. *nhaC* [51,52] and a third gene, encoding a homolgue of *napA* from *Enterococcus hirae* [53] and *Bacillus megaterium* [54], is currently under investigation. It has not clearly been shown to be involved in $Na^+$ efflux rather than participating in $K^+$ efflux as has been shown for related Kef proteins [55,56]. NhaC, the first demonstrated alkaliphile $Na^+/H^+$ antiporter [52], now has homologs and often paralogues in diverse prokaryotic genomes [57] but is yet to be assigned a major physiological role. In *B. pseudofirmus* OF4, there is a small gene expressed from an apparent operon with *nhaC* that has been designated *nhaS*. It is a putative $Na^+$ binding protein that might have roles in regulation, sensing, or raising the [$Na^+$] near the membrane (Fig. 1, [51]). Studies of a mutant with a disruption of *nhaC* in *B. pseudofirmus* OF4 led to the conclusion that in the alkaliphile, NhaC is not essential for growth at, or adaptation to, pH 10.5. However, NhaC plays a significant role at both near-neutral and high pH in the efflux of $Na^+$ when the cation concentrations are sub-optimal [51]. During a pH shift from 8.5 to 10.5, NhaC-deficient *B. pseudofirmus* OF4 regulated its cytoplasmic pH significantly less well than the wild type when the $Na^+$ concentration was, for example, 1 mM [51].

The emerging picture of the crucial $Na^+/H^+$ antiporter limb of the alkaliphile $Na^+$ cycle is that there is a dominant participant, Mrp or Sha, that may function as a complex and whose possible primary energization could be a critical piece of the alkaliphile puzzle. In addition, several secondary antiporters, including NhaC and probably at least one more [51], are supporting players in this limb of the cycle. NhaC and the other 'supporting players' may have roles, as already indicated, at relatively low [$Na^+$] or may have particular roles in assuring $Na^+$ efflux at pH 7.5. As discussed below, there is a somewhat paradoxical requirement by *B. pseudofirmus* OF4 for a higher [$Na^+$] for growth at pH 7.5 than at pH 10.5. This may necessitate special antiporter accommodations as well.

### 2.3. Completion of the $Na^+$ cycle: $Na^+$/solute symporters and $Na^+$ channels

Two types of re-entry routes have been proposed for completion of the $Na^+$ cycle. These are the numerous $Na^+$/solute symporters that are major solute acquisition transporters, and the $Na^+$ channels that are evidently found in association with the $Na^+$-dependent motility of alkaliphilic *Bacillus* species [5,6]. A particularly striking early experiment [58] demonstrated the importance of $Na^+$/solute symporters in the $Na^+$ uptake limb of the $Na^+$ cycle. Cells of alkaliphilic *B. pseudofirmus* RAB (closely related to *B.*

*pseudofirmus* OF4), equilibrated at pH 8.5, were subjected to a pH shift from an external pH of 8.5 to 10.5 in the presence of: $K^+$ buffer only; or $K^+$ buffer plus either 50 mM or 2 mM added $Na^+$ with no further additions; or $K^+$ buffer with the added $Na^+$ as well as AIB (α-aminoisobutyric acid). AIB is a non-metabolizable amino acid analogue whose uptake is coupled to $Na^+$ uptake and is energized by the inwardly directed electrochemical $Na^+$ gradient. In the absence of added $Na^+$ or with only 2 mM added $Na^+$, the cytoplasmic pH rose to pH 10.5 after the shift. That is, there was no capacity for pH homeostasis. In the presence of 50 mM added $Na^+$, the cytoplasmic pH remained below 8.5 at 10 min post-shift and gradually drifted upward whereas if AIB was also present, the cytoplasmic pH did not even drift upward. More dramatic, was the finding that if both 2 mM added $Na^+$ and AIB were present, substantial pH homeostasis was observed. The results, especially given the energy-consuming effect expected of AIB transport, strongly indicated the importance of symport as a mode of $Na^+$ re-entry in support of pH homeostasis. However, as pointed out in connection with work on the alkaliphilic bacterium *Exiguobacterium aurantiacum* [59,60], alkaliphiles can acidify their cytoplasm relative to the medium even in buffers without solutes whose transport is coupled to $Na^+$. It was proposed that there might be $Na^+$ channels for $Na^+$ entry that were opened at high pH to ensure an adequate supply of $Na^+$ for the increased antiport coupled to $H^+$ accumulation. Alkaliphile motility is $Na^+$-coupled [20,61,62] and is observed only in cells growing in the high alkaline end of the pH range for growth [36,63]. This has led to the proposal [19] that a *motAB*(*XY*)/*pomABmotXY* type of channel [33,34] that is expected to function in energization of motility could also be an important $Na^+$ route in support of pH homeostasis. This is yet to be tested, a test that should be feasible when the alkaliphile *mot*/*pom* genes are identified. Another, not mutually exclusive possibility, is that one or more $Na^+$/coupled solute systems of alkaliphiles catalyze $Na^+$ uptake that is uncoupled from solute uptake at sufficiently high pH, i.e. that there is an electrogenic $Na^+$ uniport under high pH conditions mediated by these transporters. It also should be noted that many prokaryote genomes possess NtpJ- or KtrB-like proteins that are likely to be $Na^+/K^+$ symporters [64], as shown in Fig. 1. Some of these may actually be structurally related $Na^+$ channels such as the Aradopsis HKT-1 protein [21] (Fig. 1). NtpJ or HKT-1 homologs could both be effective components of the $Na^+$ re-entry limb of the alkaliphile $Na^+$ cycle. There are two homologs of NtpJ or KtrB type protein-encoding genes in the *B. halodurans* C-125 that are annotated as '$Na^+$ synthase', presumably of the *ntpJ* localization as part of a larger locus encoding an ATP-dependent $Na^+$ efflux system in *Enterococcus*, but there is no obvious operon in the alkaliphile that is comparable to the *ntp* locus [15]; rather, it is likely that the two alkaliphile gene products are $K^+$ and/or $Na^+$ uptake pathways as hypothesized in Fig. 1.

## 3. $Na^+$ stress: low [$Na^+$] and high [$Na^+$]

### 3.1. Requirement for a higher [$Na^+$] at lower pH: a hypothesis

During studies of the phenotypes of *B. pseudofirmus* OF4 mutants as a function of pH and [$Na^+$], it has been consistently observed that growth of the wild type at pH 7.5 on malate requires a higher concentration of $Na^+$ (e.g. at least 25 mM and, optimally more than 50 mM) than growth on malate at pH 10.5 (where 10 mM $Na^+$ supports an optimal growth rate that is a bit faster than that at pH 7.5) [26,51]. We have advanced the hypothesis [26] that the proteome of the facultative alkaliphiles is largely constant, both qualitatively and even quantitatively at pH 7.5 and 10.5, with many key proteins being adapted to the high pH. Thus, for example, the $Na^+$/solute symporters and antiporters that comprise the $Na^+$ cycle at pH 10.5 are most likely the same gene products that are used at pH 7.5. In this extremophile, however, they may be adapted in particular ways to the higher pH, low proton, environment. Perhaps, the $Na^+$-translocating transporters, especially the symporters, are well adapted to high pH in a way that makes them more susceptible to competitive inhibition by $H^+$ at near-neutral pH. Since protons are not functional coupling ions for the alkaliphile symporters studied to date, increased competitive inhibition would set the minimum [$Na^+$] required for growth at near-neutral pH at a higher

value than at more optimal, alkaline pH values for growth. The high [$Na^+$] required for symport might then place special demands upon the antiport complement at near-neutral pH values accounting for the particular importance of NhaC, for example, at pH 7.5 [51].

### 3.2. Primary $Na^+$ extrusion systems not coupled to $H^+$ uptake

When the [$Na^+$] concentration is at stressful levels, primary $Na^+$ extrusion, not coupled to $H^+$ uptake would be expected to maintain an optimally low cytoplasmic concentration that is consonant with the requirements of ongoing $Na^+/H^+$ antiport. Presumably, candidates for genes encoding P-type or ABC-type $Na^+$ extrusion mechanisms will emerge from alkaliphile genomics. As noted in connection with Fig. 1, the NatCAB system is the first ABC-type $Na^+$ extrusion system to be identified [23]. A homologous system in *B. subtilis*, NatAB, is modestly inducible by agents such as ethanol and protonophores, that lower the protonmotive force across the membrane [65]. The boost in the $\Delta\Psi$ that is then achieved via NatAB-dependent $Na^+$ extrusion apparently energizes $K^+$ acquisition by some independent pathway. NatAB thereby functions directly in $Na^+$-resistance and further provides an ATP-dependent increase in the $\Delta\Psi$ that can support important secondary transporters. The interplay between $Na^+$ energetics and $K^+$ homeostasis in the alkaliphilic *Bacillus* species has not yet been studied. In this context, the possibility of genes encoding a primary system in *B. halodurans* C-125, that are not organized in an operon, may be more readily explored now that the genome sequence is available [15].

## 4. Interplay of the '$Na^+$ profile' with properties of cell surface layers

The possible role of specific membrane lipids in alkaliphile energetics, including $Na^+$ as well as $H^+$ binding and permeability, has not yet been clarified. Membrane lipids of alkaliphilic *Bacillus* species have been analyzed, but not in an experimental context that explored a $Na^+$ linkage. In liposomes prepared from lipids of the haloalkaliphilic archaeal organism, *Halorubrum vacuolatum*, van de Vossenberg et al. [66] showed that the proton permeability did not change in a pH-dependent fashion but did increase with increasing temperature. $Na^+$ permeability was shown to increase with increased [$Na^+$]. The properties of the lipid phase of the alkaliphile coupling membrane in relation to key problems of alkaliphily remains an under-explored area.

More headway has been made in evaluating the contributions of Secondary Cell Wall Polymers (SCWP) to alkaliphile growth and/or pH homeostasis at high pH. The first proposals of a significant role for such polymers came primarily from the work of Aono and colleagues [24,25,67,68]. They showed that the high negative surface charge noted in alkaliphilic *Bacillus* strains could - for some classes of *Bacillus* but not others - be attributed to a teichuronic acid containing N-acetyl-D-fucosamine, glucuronic acid and galacturonic acid and a teichuronopeptide consisting of a polyglutamate polymer and a polyglucuronic acid polymer [69–71]. Mutants defective in these polymers [23,67,68], especially the teichuronopeptide [25], exhibit defective growth and pH homeostasis at high pH. However, as assessed by the mutant studies, these polymers are not essential for alkaliphilic growth or pH homeostasis [25]. Nor is it clear whether it is the adjustment to growth at high pH or actual logarithmic growth at the upper end of the pH range that is primarily affected. In *B. pseudofirmus* OF4, recent work has revealed an S-layer (surface layer) protein, SlpA, that forms a crystalline array on the cell surface [26]. Loss of this layer accompanied disruption of the *slpA* gene. The *slpA*-minus mutant, RG21, grows better than the wild type at pH 7.5 (with a shorter lag and/or faster logarithmic growth rate, depending upon the $Na^+$ concentration). This suggests that the energetic burden of S-layer production by the alkaliphile at pH 7.5 is not offset by a net benefit to the cell at that pH. The mutant grows almost identically to the wild type during the logarithmic phase of growth at pH 10.5 but exhibits a longer lag, especially at sub-optimal $Na^+$ concentrations or further elevation of the pH to 11; a defect in pH homeostasis upon a sudden shift from pH 8.5 to either pH 10.5 or 11 was also noted [26]. The S-layer of *B. pseudofirmus* OF4 seems to be constitutively formed as a major cell surface

protein even though the cost of its production is apparently disadvantageous at pH 7.5. Presumably, the alkaliphile offsets this disadvantage by the preparedness conferred by SlpA for a sudden upward shift in external pH. A gene locus that would putatively encode a polyglutamate capsule has also been partially characterized in *B. pseudofirmus* OF4 [72] but there is no evidence yet that such a capsule is actually made, let alone is of significance with respect to alkaliphily. It will be of interest, to pursue this question as well as whether *B. pseudofirmus* OF4 and *B. halodurans* C-125 have additional SCWP layers that have a role in adaptation to, or growth at, very high pH.

## 5. Concluding remarks

The next few years should provide opportunities for the application of sophisticated genomics to studies of the extremely alkaliphilic *Bacillus* species. This should help identify the candidates for primary and secondary transporters that participate in $Na^+$-dependent processes that are key to alkaliphily and in resistance to potentially toxic levels of $Na^+$. Further clarification of the mechanism and extent of passive adjuncts to $Na^+$-related physiology, e.g. SCWPs or special cytoplasmic binding proteins or buffers, may also be clarified. Most importantly, more detailed molecular and biochemical studies of the major transporters (and channels) of the $Na^+$ cycle that supports the remarkable pH homeostasis of these alkaliphiles should clarify the mechanism by which the alkaliphilic *Bacillus* species meet this central challenge.

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